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<p>(54) Title: A METHOD OF PREPARING A VARIANT OF A LIPOLYTIC ENZYME</p> <p>(57) Abstract A method of preparing a variant of a parent lipolytic enzyme, which method comprises: (a) subjecting a DNA sequence encoding the parent lipolytic enzyme to random mutagenesis, (b) expressing the mutated DNA sequence obtained in step (a) in a host cell, and (c) screening for host cells expressing a mutated lipolytic enzyme which has a decreased dependance to calcium and/or an improved tolerance towards a detergent or a detergent component as compared to the parent lipolytic enzyme.</p>		

## A METHOD OF PREPARING A VARIANT OF A LIPOLYTIC ENZYME

## FIELD OF THE INVENTION

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The present invention relates to a method of preparing a variant of a parent lipolytic enzyme and to variants prepared by the method. Furthermore, the invention relates to a DNA construct encoding a variant of the invention, an expression  
10 vector and host cell comprising the DNA construct and a detergent additive or a detergent composition comprising a variant.

## BACKGROUND OF THE INVENTION

15

For a number of years lipolytic enzymes have been used as detergent enzymes, i.e. to remove lipid or fatty stains from clothes and other textiles.

20 For instance, various microbial lipases have been suggested as detergent enzymes. Examples of such lipases include a *Humicola lanuginosa* lipase, e.g. described in EP 258 068 and EP 305 216, a *Rhizomucor miehei* lipase, e.g. as described in EP 238 023, a *Candida* lipase, such as a *C. antarctica* lipase, e.g. the *C.*  
25 *antarctica* lipase A or B described in EP 214 761, a *Pseudomonas* lipase such as a *P. alcaligenes* and *P. pseudoalcaligenes* lipase, e.g. as described in EP 218 272, a *P. cepacia* lipase, e.g. as described in EP 331 376, a *Bacillus* lipase, e.g. a *B. subtilis* lipase (Dartois et al., 1993), a *B. stearothermophilus*  
30 lipase (JP 64/744992) and a *B. pumilus* lipase (EP 91 00664).

Furthermore, a number of cloned lipases have been described, including the *Penicillium camembertii* lipase described by Yamaguchi, S. et al., 1991, the *Geotricum candidum* lipase  
35 (Schimada, Y. et al., 1989), and various *Rhizopus* lipases such as a *R. delemar* lipase (Hass, M.J et al., 1991), a *R. niveus* lipase (Kugimiya, W. 1992), and a *R. oryzae* lipase.

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A need exists for novel lipolytic enzymes having improved washing and/or dishwashing properties, and the object of the present invention is to prepare such enzymes.

#### 5 BRIEF DISCLOSURE OF THE INVENTION

The present inventors have now developed a novel method of preparing variants of lipolytic enzymes having improved washing and/or dishwashing performance as compared to their parent en-  
10 zymes. The method is based on random or localized random mutagenesis of DNA sequences encoding a lipolytic enzyme.

More specifically, in a first aspect the invention relates to a method of preparing a variant of a parent lipolytic enzyme,  
15 which method comprises

- (a) subjecting a DNA sequence encoding the parent lipolytic enzyme to random mutagenesis,
- 20 (b) expressing the mutated DNA sequence obtained in step (a) in a host cell, and
- (c) screening for host cells expressing a mutated lipolytic enzyme which has a decreased dependance to calcium and/or an  
25 improved tolerance towards a detergent or one or more detergent components as compared to the parent lipolytic enzyme.

In the present context, the term "lipolytic enzyme" is intended to indicate an enzyme exhibiting a lipid degrading capability,  
30 such as a capability of degrading a triglycerid or a phospholipid. The lipolytic enzyme may, e.g., be a lipase, a phospholipase, an esterase or a cutinase.

The term "random mutagenesis" is intended to be understood in  
35 a conventional manner, i.e. to indicate an introduction of one or more mutations at random positions of the parent enzyme (i.e. as opposed to site-specific mutagenesis). The random mutations are typically introduced by exposing a large number

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The screening criteria defined in step c) of the method of the invention may be determined by any suitable methods known in the art. A particular suitable assay developed for the present purpose is described in the Materials and Methods section  
5 below.

In a further aspect the invention relates to a DNA construct comprising a mutated DNA sequence encoding a variant of a lipolytic enzyme which has a decreased dependance to calcium and/or  
10 an improved tolerance towards a detergent or a detergent component as compared to the parent lipolytic enzyme, which DNA sequence is isolated from the host cell selected in step (c) of the method of the invention.

15 In a still further aspect the invention relates to a recombinant expression vector carrying the DNA construct, a cell which is transformed with the DNA construct or the vector as well as a method of producing the variant of the parent lipolytic enzyme by culturing said cell under conditions conducive to the  
20 production of the variant, after which the variant is recovered from the culture.

In final aspects the invention relates to a variant of a lipolytic enzyme and the use of said variant as a detergent  
25 enzyme, in particular for washing or dishwashing, and to a detergent additive and a detergent composition comprising the variant.

### 30 DETAILED DISCLOSURE OF THE INVENTION

#### **Cloning a DNA sequence encoding a parent lipolytic enzyme**

The DNA sequence encoding a parent lipolytic enzyme to be subjected to random mutagenesis in accordance with the present invention may be isolated from any cell or microorganism producing the parent enzyme in question by use of methods known in  
35 the art.

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**Random mutagenesis**

The random mutagenesis of the DNA sequence encoding the parent lipolytic enzyme to be performed in accordance with step a) of the method of the invention may conveniently be performed by  
5 use of any method known in the art.

For instance, the random mutagenesis may be performed by use of a suitable physical or chemical mutagenizing agent, by use of a suitable oligonucleotide, or by subjecting the DNA sequence  
10 to PCR generated mutagenesis. Furthermore, the random mutagenesis may be performed by use of any combination of these mutagenizing agents.

The mutagenizing agent may, e.g., be one which induces tran-  
15 sitions, transversions, inversions, scrambling, deletions, and/or insertions.

Examples of a physical or chemical mutagenizing agent suitable for the present purpose includes ultraviolet (UV) irradiation,  
20 hydroxylamine, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), O-methyl hydroxylamine, nitrous acid, ethyl methane sulphonate (EMS), sodium bisulphite, formic acid, and nucleotide analogues.

25 When such agents are used the mutagenesis is typically performed by incubating the DNA sequence encoding the parent enzyme to be mutagenized in the presence of the mutagenizing agent of choice under suitable conditions for the mutagenesis to take place, and selecting for mutated DNA having the desired  
30 properties.

When the mutagenesis is performed by the use of an oligonucleotide, the oligonucleotide may be doped or spiked with the three non-parent nucleotides during the synthesis of the oligonucleo-  
35 tide at the positions wanted to be changed. The doping or spiking may be done so that codons for unwanted amino acids are avoided. The doped or spiked oligonucleotide can be incorporated into the DNA encoding the lipolytic enzyme by any

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Subsequent to the incubation with or exposure to the mutagenizing agent, the mutated DNA is expressed by culturing a suitable host cell carrying the DNA sequence under conditions allowing expression to take place. The host cell used for this purpose may be one which has been transformed with the mutated DNA sequence, optionally present on a vector, or one which was carried the DNA sequence encoding the parent enzyme during the mutagenesis treatment. Examples of suitable host cells are given below. The mutated DNA sequence may further comprise a DNA sequence encoding functions permitting expression of the mutated DNA sequence.

It will be understood that the screening criteria mentioned in step (c) above have been carefully selected. Thus, without being limited to any theory the screening for a decreased dependency to calcium is believed to result in variants having an over-all improved performance in that the requirement for calcium may be considered a limiting factor for optimal activity, in particular under conditions where only low amounts of free calcium ions are present. In connection with detergent lipases the free calcium ions required are normally provided from the washing water and thus, the lipolytic activity is dependent on the calcium content of the water.

The detergent or detergent component towards which the variant has improved tolerance may be of any type, e.g. as further described below. Preferably, the detergent component is a non-ionic, anionic, cationic, zwitterionic or amphoteric surfactant. Examples of non-ionic surfactants include an alcohol ethoxylate, examples of anionic surfactants include LAS, alkyl sulphate, alcohol ethoxy sulphate and the like.

In particular, it is contemplated that an improved tolerance towards a non-ionic surfactant alcohol ethoxylate, a commercially available example of which is Dobanol®, may be indicative of improved wash performance.

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any detergent composition in combination with one of the above detectors of enzymatic activity.

It will be understood that the screening criteria used in the filter assay of the invention may be chosen so as to comply with the desired properties or uses of the enzymes to be screened. For instance, in a screening for lipases of particular use in the paper and pulp industry, it may be relevant to screen for an acid lipase having an increased temperature stability. This may be performed by using a buffer with acidic pH (e.g. pH 4) and/or incubate under higher temperature before or under the assay.

The host cells produced in step (c) may be subjected to further rounds of mutagenesis as defined in steps (a)-(c) above, conveniently by using more stringent selection criteria than employed in a previous mutagenesis treatment.

The host cells selected for in step (c) may be used directly for the production of the variant of the lipolytic enzyme. Alternatively, DNA encoding the variant may be isolated from the host cell and inserted into another suitable host cell, conveniently by use of the procedure described below in the section entitled "Expression of a variant of the invention", in which suitable host cells are also listed.

#### Localized random mutagenesis

In accordance with the invention the random mutagenesis may advantageously be located to a part of the parent lipolytic enzyme in question. This may, e.g., be advantageous when a certain region of the enzyme has been identified to be of particular importance for a given property of the enzyme, and which, when modified, is expected to result in a variant having improved properties. Such region may normally be identified when the tertiary structure of the parent enzyme has been elucidated and related to the function of the enzyme.

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during hydrolysis where the lipase hydrolyses triglycerides from the lipid phase when activated by contact with the lipid surface.

5 The lipid contact zone contains a binding area for the lipid substrate which is the part of the lipid contact zone to which the single lipid substrate molecule binds before hydrolysis. This binding area again contains an acyl-binding hydrophobic cleft and a so-called hydrolysis pocket, which is situated  
10 around the active site Ser, and in which the hydrolysis of the lipid substrate is believed to take place. In all lipases known today the lipid contact zone is easily recognized, e.g. from a three-dimensional structure of the lipase created by suitable computer programs. The conformation of an inactive and acti-  
15 vated lipase, respectively, is shown in Figs. 1 and 2 of WO 92/05249.

The lipid contact zone of the *Humicola lanuginosa* lipase discussed in detail in the present application is defined by amino  
20 acid residues 21-25, 36-38, 56-62, 81-98, 110-116, 144-147, 172-174, 199-213 and 248-269. These residues have been identified on the basis of computer model simulations of the interaction between the lipase and a lipid substrate.

- 25 The lipid contact zone of other lipolytic enzymes is defined by
- a) calculating the hydrophobic vector of the 3-D molecular structure,
  - b) making a cut perpendicular to the vector through the C $\alpha$ -  
30 atom of the second amino acid residue after the active site serine in the linear sequence, and
  - c) including all residues with at least one atom on that side of the cut to which the vector points, and
  - d) selecting from those residues, those which have at least  
35 one atom within 5 Ångström of the surface of the protein (in case of a lipase in either its open or closed form).

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question, but also an enzyme encoded by a DNA sequence isolated from such strain and produced in a host organism transformed with said DNA sequence. Furthermore, the term is intended to indicate an enzyme which is encoded by a DNA sequence of 5 synthetic and/or cDNA origin and which has the identifying characteristics of the enzyme in question.

Of particular interest as a parent lipolytic enzyme is a lipase derivable from a strain of *H. lanuginosa*, e.g. the *H. lanu-*  
10 *ginosa* strain DSM 4109, or an analogue of said lipase, a strain of *Rh. mucor*, or a strain of *C. antarctica*.

In the present context the term "analogue" is intended to include a polypeptide which comprises an amino acid sequence 15 differing from that of the *H. lanuginosa* lipase by one or more amino acid residues, and which is at least 70% homologous with the amino acid sequence of said lipase, (determined as the degree of identity between the two sequences), such as at least 75%, 80%, 90% or 95% homologous, is immunologically cross  
20 reactive with said lipase, and/or which is encoded by a DNA sequence hybridizing with an oligo nucleotide probe prepared on the basis of the amino acid sequence of said lipase or of a DNA sequence encoding said lipase.

25 The analogue may be a derivative of the *H. lanuginosa* lipase, e.g. prepared by modifying a DNA sequence encoding the lipase resulting in the addition of one or more amino acid residues to either or both the N- and C-terminal end of the lipase, substi-  
30 tution of one or more amino acid residues at one or more different sites in the amino acid sequence, deletion of one or more amino acid residues at either or both ends of the lipase or at one or more sites in the amino acid sequence, or insertion of one or more amino acid residues at one or more sites in the amino acid sequence. The modification of the DNA  
35 sequence may be performed by site-directed or by random mutagenesis or a combination of these techniques in accordance with well-known procedures.

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The localized random mutagenesis may be performed in one or more of these regions, and is preferably performed in at least two of the regions.

- 5 The parent lipolytic enzyme to be modified in accordance with the present invention may be derivable from a bacterium. For instance, the DNA sequence encoding the parent lipolytic enzyme may be derivable from a strain of *Pseudomonas* spp., such as *P. cepacia*, *P. alcaligenes*, *P. pseudoalcaligenes*, *P. mendocina*  
10 (also termed *P. putida*), *P. syringae*, *P. aeruginosa* or *P. fragi*, a strain of *Bacillus* spp., e.g. *B. subtilis* or *B. pumilus* or a strain of *Streptomyces* sp., e.g. *S. scabies*.

The parent bacterial lipolytic enzyme may be a lipase derived  
15 from any of the above-mentioned species, e.g. a *Pseudomonas* lipase as described in EP 218 272, EP 331 376 and EP 407 225, or a cutinase, e.g. as described in WO 88/09367.

#### Variants of the invention

- 20 For ease of reference specific variants of the invention are described by use of the following nomenclature:

Original amino acid(s):position(s):substituted amino acid(s)

- 25 According to this nomenclature, for instance the substitution of aspartic acid for valine in position 96 is shown as:

Asp 96 Val or D96V

a deletion of aspartic acid in the same position is shown as:

Asp 96 \* or D96\*

- 30 and insertion of an additional amino acid residue such as lysine is shown as:

Asp 96 ValLys or D96VK

Multiple mutations are separated by pluses, i.e.:

- 35 Asp 96 Val + Glu 87 Lys or D96V+E87K

representing mutations in positions 96 and 87 substituting aspartic acid and glutamic acid for valine and lysine, respectively.

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In addition the invention relates to a variant of the *H. lanuginosa* lipase obtainable from DSM 4109 or an analogue of said lipase, wherein the amino acid residue L264 has been replaced by an amino acid different from Leucine, i.e. any one  
5 of R, N, A, C, Q, E, G, H, I, K, M, F, P, S, T, W, Y, V, D.

Preferably, the variant according to the invention comprises at least one of the following mutations K46R, E57G, G61S, S83T, S58F, D62C, T64R, I90F, G91A, N92H, N94I, N94K, L97M, K98I,  
10 I100V, D102K, A121V, E129K, D167G, R205K, E210W, K237M, N259W, I252L, D254W, P256T, G263A, L264Q or T267W.

These positions have been found or is contemplated to be important for enzymatic activity and/or detergent tolerance. The  
15 numbering of the amino acid residues refers to the amino acid sequence of the mature lipase.

Preferably, the variant according to this aspect of the invention comprises at least one of the following mutations  
20 S83T, N94K, A121V, D167G, R205K.

It will be understood that the present invention encompasses variants of the parent *H. lanuginosa* lipase comprising a combination of two or more of the mutations defined herein, or  
25 a combination of one or more of the mutations defined herein with any of the mutations disclosed in WO 92/05249, WO 94/25577 and WO 94/01541.

In a further aspect the present invention relates to a variant  
30 of the *H. lanuginosa* lipase obtainable from DSM 4109 or an analogue thereof comprising at least one of the following mutations:

N94K+D96A

35 S83T+N94K+D96N

E87K+D96V

E87K+G91A+D96A

N94K+F95L+D96H

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constructing these variants would be based on site-directed mutagenesis using suitable oligonucleotide probes. This method is exemplified in Examples 3-6.

#### 5 Expression of a variant of the invention

According to the invention, a mutated DNA sequence encoding a variant lipolytic enzyme prepared by methods described above, or any alternative methods known in the art, can be expressed, in enzyme form, using an expression vector which typically  
10 includes control sequences encoding a promoter, operator, ribosome binding site, translation initiation signal, and, optionally, a repressor gene or various activator genes.

The recombinant expression vector carrying the DNA sequence  
15 encoding a variant of the invention may be any vector which may conveniently be subjected to recombinant DNA procedures, and the choice of vector will often depend on the host cell into which it is to be introduced. Thus, the vector may be an autonomously replicating vector, i.e. a vector which exists as  
20 an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid, a bacteriophage or an extrachromosomal element, minichromosome or an artificial chromosome. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the  
25 host cell genome and replicated together with the chromosome(s) into which it has been integrated.

In the vector, the DNA sequence should be operably connected to a suitable promoter sequence. The promoter may be any DNA  
30 sequence which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell. Examples of suitable promoters for directing the transcription of the DNA sequence encoding a variant of the invention, especially in a  
35 bacterial host, are the promoter of the *lac* operon of *E.coli*, the *Streptomyces coelicolor* agarase gene *dagA* promoters, the promoters of the *Bacillus licheniformis*  $\alpha$ -amylase gene (*amyL*), e.g. as described in WO 93/10249 the promoters of the *Bacillus*

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medium. If desirable, this preregion may be replaced by a different preregion or signal sequence, convenient accomplished by substitution of the DNA sequences encoding the respective preregions.

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The procedures used to ligate the DNA construct of the invention encoding a variant of a parent lipolytic enzyme, the promoter, terminator and other elements, respectively, and to insert them into suitable vectors containing the information necessary for replication, are well known to persons skilled in the art (cf., for instance, Sambrook et al. (1989)).

The cell of the invention either comprising a DNA construct or an expression vector of the invention as defined above is advantageously used as a host cell in the recombinant production of a variant of a parent lipolytic enzyme of the invention. The cell may be transformed with the DNA construct of the invention encoding the variant, conveniently by integrating the DNA construct in the host chromosome. This integration is generally considered to be an advantage as the DNA sequence is more likely to be stably maintained in the cell. Integration of the DNA constructs into the host chromosome may be performed according to conventional methods, e.g. by homologous or heterologous recombination. Alternatively, the cell may be transformed with an expression vector as described below in connection with the different types of host cells.

The cell of the invention may be a cell of a higher organism such as a mammal or an insect, but is preferably a microbial cell, e.g. a bacterial or a fungal (including yeast) cell.

Examples of suitable bacteria are grampositive bacteria such as *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus lentus*, *Bacillus brevis*, *Bacillus stearothermophilus*, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus coagulans*, *Bacillus circulans*, *Bacillus lautus*, *Bacillus megaterium*, *Bacillus thuringiensis*, or *Streptomyces lividans* or *Streptomyces murinus*, or gramnegative bacteria such as *E.coli*. The trans-

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Due to the decreased dependance to calcium and/or improved tolerance towards detergents or detergent components of the variant of the invention, the variant is particularly well suited for implementation into detergent compositions, e.g. 5 detergent compositions intended for performance in the range of pH 7-13, particularly the range of pH 8-11.

#### Detergent Compositions

10 According to the invention, a lipase variant of the invention may typically be a component of a detergent composition. As such, it may be included in the detergent composition in the form of a non-dusting granulate, a stabilized liquid, or a protected enzyme. Non-dusting granulates may be produced, e.g., 15 as disclosed in US 4,106,991 and 4,661,452 (both to Novo Industri A/S) and may optionally be coated by methods known in the art. Examples of waxy coating materials are poly(ethylene oxide) products (polyethyleneglycol, PEG) with mean molecular weights of 1000 to 20000; ethoxylated nonylphenols having from 20 16 to 50 ethylene oxide units; ethoxylated fatty alcohols in which the alcohol contains from 12 to 20 carbon atoms and in which there are 15 to 80 ethylene oxide units; fatty alcohols; fatty acids; and mono- and di- and triglycerides of fatty acids. Examples of film-forming coating materials suitable for 25 application by fluid bed techniques are given in patent GB 1483591. Liquid enzyme preparations may, for instance, be stabilized by adding a polyol such as propylene glycol, a sugar or sugar alcohol, lactic acid or boric acid according to established methods. Other enzyme stabilizers are well known in 30 the art. Protected enzymes may be prepared according to the method disclosed in EP 238,216.

The detergent composition of the invention may be in any convenient form, e.g. as powder, granules, paste or liquid. A 35 liquid detergent may be aqueous, typically containing up to 70% water and 0-30% organic solvent, or nonaqueous.

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tetraacetythylenediamine (TAED) or nonanoyloxybenzene-sulfonate (NOBS). Alternatively, the bleaching system may comprise peroxyacids of, e.g., the amide, imide, or sulfone type.

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The enzymes of the detergent composition of the invention may be stabilized using conventional stabilizing agents, e.g. a polyol such as propylene glycol or glycerol, a sugar or sugar alcohol, lactic acid, boric acid, or a boric acid derivative  
10 such as, e.g., an aromatic borate ester, and the composition may be formulated as described in, e.g., WO 92/19709 and WO 92/19708.

The detergent may also contain other conventional detergent  
15 ingredients such as, e.g., fabric conditioners including clays, foam boosters, suds suppressors, anti-corrosion agents, soil-suspending agents, anti-soil-redeposition agents, dyes, bactericides, optical brighteners, or perfume.

20 The pH (measured in aqueous solution at use concentration) will usually be neutral or alkaline, e.g. in the range of 7-11.

Particular forms of detergent compositions within the scope of the invention include:

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(2) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

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	Linear alkylbenzenesulfonate (calculated as acid)	6 - 11%
10	Alcohol ethoxysulfate (e.g. C <sub>12-18</sub> alcohol, 1-2 EO or alkyl sulfate (e.g. C <sub>16-18</sub> ))	1 - 3%
	Alcohol ethoxylate (e.g. C <sub>14-15</sub> alcohol, 7 EO)	5 - 9%
	Sodium carbonate (as Na <sub>2</sub> CO <sub>3</sub> )	15 - 21%
15	Soluble silicate (as Na <sub>2</sub> O, 2SiO <sub>2</sub> )	1 - 4%
	Zeolite (as NaAlSiO <sub>4</sub> )	24 - 34%
	Sodium sulfate (as Na <sub>2</sub> SO <sub>4</sub> )	4 - 10%
	Sodium citrate/citric acid (as C <sub>6</sub> H <sub>5</sub> Na <sub>3</sub> O <sub>7</sub> /C <sub>6</sub> H <sub>8</sub> O <sub>7</sub> )	0 - 15%
20	Carboxymethylcellulose	0 - 2%
	Polymers (e.g. maleic/acrylic acid copolymer, PVP, PEG)	1 - 6%
	Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
25	Minor ingredients (e.g. suds suppressors, perfume)	0 - 5%

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(4) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

5	Linear alkylbenzenesulfonate (calculated as acid)	8	- 12%
	Alcohol ethoxylate (e.g. C <sub>12-15</sub> alcohol, 7 EO)	10	- 25%
10	Sodium carbonate (as Na <sub>2</sub> CO <sub>3</sub> )	14	- 22%
	Soluble silicate (as Na <sub>2</sub> O, 2SiO <sub>2</sub> )	1	- 5%
	Zeolite (as NaAlSiO <sub>4</sub> )	25	- 35%
	Sodium sulfate (as Na <sub>2</sub> SO <sub>4</sub> )	0	- 10%
	Carboxymethylcellulose	0	- 2%
15	Polymers (e.g. maleic/acrylic acid copolymer, PVP, PEG)	1	- 3%
	Enzymes (calculated as pure enzyme protein)	0.0001	- 0.1%
20	Minor ingredients (e.g. suds suppressors, perfume)	0	- 5%

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(6) An aqueous structured liquid detergent composition comprising

5	Linear alkylbenzenesulfonate (calculated as acid)	15	- 21%
	Alcohol ethoxylate (e.g. C <sub>12-15</sub> alcohol, 7 EO, or C <sub>12-15</sub> alcohol, 5 EO)	3	- 9%
10	Soap as fatty acid (e.g. oleic acid)	3	- 10%
	Zeolite (as NaAlSiO <sub>4</sub> )	14	- 22%
	Potassium citrate	9	- 18%
	Borate (as B <sub>4</sub> O <sub>7</sub> )	0	- 2%
15	Carboxymethylcellulose	0	- 2%
	Polymers (e.g. PEG, PVP)	0	- 3%
	Anchoring polymers such as, e.g., lauryl methacrylate/acrylic acid copolymer; molar ratio 25:1; MW 3800	0	- 3%
20	Glycerol	0	- 5%
	Enzymes (calculated as pure enzyme protein)	0.0001	- 0.1%
25	Minor ingredients (e.g. dispersants, suds suppressors, perfume, optical brighteners)	0	- 5%

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(8) A detergent composition formulated as a granulate comprising

5	Linear alkylbenzenesulfonate (calculated as acid)	8	- 14%
	Ethoxylated fatty acid monoethanol- amide	5	- 11%
	Soap as fatty acid	0	- 3%
10	Sodium carbonate (as $\text{Na}_2\text{CO}_3$ )	4	- 10%
	Soluble silicate (as $\text{Na}_2\text{O}, 2\text{SiO}_2$ )	1	- 4%
	Zeolite (as $\text{NaAlSiO}_4$ )	30	- 50%
	Sodium sulfate (as $\text{Na}_2\text{SO}_4$ )	3	- 11%
	Sodium citrate (as $\text{C}_6\text{H}_7\text{Na}_3\text{O}_7$ )	5	- 12%
15	Polymers (e.g. PVP, maleic/acrylic acid copolymer, PEG)	1	- 5%
	Enzymes (calculated as pure enzyme protein)	0.0001	- 0.1%
20	Minor ingredients (e.g. suds suppressors, perfume)	0	- 5%

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(10) An aqueous liquid detergent composition comprising

5	Linear alkylbenzenesulfonate (calculated as acid)	15	- 23%
	Alcohol ethoxysulfate (e.g. C <sub>12-15</sub> alcohol, 2-3 EO)	8	- 15%
10	Alcohol ethoxylate (e.g. C <sub>12-15</sub> al- cohol, 7 EO, or C <sub>12-15</sub> alcohol, 5 EO)	3	- 9%
	Soap as fatty acid (e.g. lauric acid)	0	- 3%
	Aminoethanol	1	- 5%
	Sodium citrate	5	- 10%
15	Hydrotrope (e.g. sodium toluenesulfonate)	2	- 6%
	Borate (as B <sub>4</sub> O <sub>7</sub> )	0	- 2%
	Carboxymethylcellulose	0	- 1%
	Ethanol	1	- 3%
20	Propylene glycol	2	- 5%
	Enzymes (calculated as pure enzyme protein)	0.0001	- 0.1%
25	Minor ingredients (e.g. polymers, dispersants, perfume, optical brighteners)	0	- 5%

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(12) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

5	Anionic surfactant (linear alkylbenzenesulfonate, alkyl sulfate, alpha-olefinsulfonate, alpha-sulfo fatty acid methyl esters, alkanesulfonates, soap)	25	- 40%
10	Nonionic surfactant (e.g. alcohol ethoxylate)	1	- 10%
	Sodium carbonate (as $\text{Na}_2\text{CO}_3$ )	8	- 25%
	Soluble silicates (as $\text{Na}_2\text{O}$ , $2\text{SiO}_2$ )	5	- 15%
	Sodium sulfate (as $\text{Na}_2\text{SO}_4$ )	0	- 5%
15	Zeolite (as $\text{NaAlSiO}_4$ )	15	- 28%
	Sodium perborate (as $\text{NaBO}_3 \cdot 4\text{H}_2\text{O}$ )	0	- 20%
	Bleach activator (TAED or NOBS)	0	- 5%
	Enzymes (calculated as pure enzyme protein)	0.0001	- 0.1%
20	Minor ingredients (e.g. perfume, optical brighteners)	0	- 3%

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(15) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

5	(C <sub>12</sub> -C <sub>18</sub> ) alkyl sulfate	4	- 8%
	Alcohol ethoxylate	11	- 15%
	Soap	1	- 4%
	Zeolite MAP or zeolite A	35	- 45%
	Sodium carbonate (as Na <sub>2</sub> CO <sub>3</sub> )	2	- 8%
10	Soluble silicate (as Na <sub>2</sub> O, 2SiO <sub>2</sub> )	0	- 4%
	Sodium percarbonate	13	- 22%
	TAED	1	- 8%
	Carboxymethyl cellulose	0	- 3%
15	Polymers (e.g. polycarboxylates and PVP)	0	- 3%
	Enzymes (calculated as pure enzyme protein)	0.0001	- 0.1%
20	Minor ingredients (e.g. optical brightener, phosphonate, perfume)	0	- 3%

(16) Detergent formulations as described in 1) - 15) which contain a stabilized or encapsulated peracid, either as an additional component or as a substitute for already specified bleach systems.

25

(17) Detergent compositions as described in 1), 3), 7), 9) and 12) wherein perborate is replaced by percarbonate.

(18) Detergent compositions as described in 1), 3), 7), 9), 30 12), 14) and 15) which additionally contain a manganese catalyst. The manganese catalyst may, e.g., be one of the compounds described in "Efficient manganese catalysts for low-temperature bleaching", Nature 369, 1994, pp. 637-639.

35 (19) Detergent composition formulated as a nonaqueous detergent liquid comprising a liquid nonionic surfactant such as, e.g., linear alkoxyated primary alcohol, a builder system (e.g.

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polycarboxylates, polyacetyl carboxylates and polyhydroxysulphonates.

Other suitable organic builders include the higher molecular weight polymers and co-polymers known to have builder properties, for example appropriate polyacrylic acid, polymaleic and polyacrylic/polymaleic acid copolymers and their salts.

The dishwashing detergent composition may contain bleaching agents of the chlorine/bromine-type or the oxygen-type. Examples of inorganic chlorine/bromine-type bleaches are lithium, sodium or calcium hypochlorite and hypobromite as well as chlorinated trisodium phosphate. Examples of organic chlorine/bromine-type bleaches are heterocyclic N-bromo and N-chloro imides such as trichloroisocyanuric, tribromoisocyanuric, dibromoisocyanuric and dichloroisocyanuric acids, and salts thereof with water-solubilizing cations such as potassium and sodium. Hydantoin compounds are also suitable.

The oxygen bleaches are preferred, for example in the form of an inorganic persalt, preferably with a bleach precursor or as a peroxy acid compound. Typical examples of suitable peroxy bleach compounds are alkali metal perborates, both tetrahydrates and monohydrates, alkali metal percarbonates, per silicates and perphosphates. Preferred activator materials are TAED and glycerol triacetate.

The dishwashing detergent composition of the invention may be stabilized using conventional stabilizing agents for the enzyme(s), e.g. a polyol such as e.g. propylene glycol, a sugar or a sugar alcohol, lactic acid, boric acid, or a boric acid derivative, e.g. an aromatic borate ester.

The dishwashing detergent composition may also comprise other enzymes, in particular an amylase, a protease and/or a cellulase.

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The invention is further described in the following examples which are not, in any way, intended to limit the scope of the invention as claimed.

5

#### MATERIALS AND METHODS

Humicola lanuginosa DSM 4109 available from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH,  
10 Mascheroderweg 1b, D-3300 Braunschweig, Federal Republic of Germany.

pYESHL is a yeast/*E. coli* shuttle vector that expresses and secretes a low level of the *H. lanuginosa* lipase in yeast. More  
15 specifically pYESHL is a derivative of pYES2 (purchased from Invitrogen Corp., UK) in which the GAL1 promoter was excised and the *Humicola lanuginosa* lipase gene and the TPI (triose phosphate isomerase) promoter from *S. cerevisiae* (Alber, T. and Kawasaki, G., *J.Mol.Appl. Genet* 1, 419-434 (1982) were cloned  
20 between the SphI and XbaI sites. A restriction map of pYESHL is shown in Fig. 1.

#### Low calcium filter assay

##### Procedure

- 25 1) Provide SC Ura<sup>r</sup> replica plates (useful for selecting strains carrying the expression vector) with a first protein binding filter (Nylon membrane) and a second low protein binding filter (Cellulose acetate) on the top.
- 30 2) Spread yeast cells containing a parent lipase gene or a mutated lipase gene on the double filter and incubate for 2 or 3 days at 30°C.
- 3) Keep the colonies on the top filter by transferring the  
35 topfilter to a new plate.
- 4) Remove the protein binding filter to an empty petri dish.

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b) *Performing localized random mutagenesis*

A mutagenic primer (oligonucleotide) is synthesized which corresponds to the part of the DNA sequence to be mutagenized except for the nucleotide(s) corresponding to amino acid s codon(s) to be mutagenized.

Subsequently, the resulting mutagenic primer is used in a PCR reaction with a suitable opposite primer. The resulting PCR fragment is purified and digested and cloned into the shuttle  
10 vector. Alternatively and if necessary, the resulting PCR fragment is used in a second PCR reaction as a primer with a second suitable opposite primer so as to allow digestion and cloning of the mutagenized region into the shuttle vector. The PCR reactions are performed under normal conditions.

15

DNA sequencing was performed by using applied Biosystems ABI DNA sequence model 373A according to the protocol in the ABI Dye Terminator Cycle Sequencing kit.

20

EXAMPLES

EXAMPLE 1

25 **Construction of random lipase variants**

Random mutagenized libraries of the entire *H. lanuginosa* lipase gene and of amino acids (aa) 91-97 and 206-211 thereof were prepared as described in Materials and Methods above.

30 The amino acid regions 91-97 and 206-211 were chosen for the first round of localized mutagenesis since these regions have been found to be important for wash performance. Region 91-97 is a part of the lid region of the lipase and region 206-211 constitutes part of the hydrophobic cleft of the lipase.

35

One oligonucleotide was synthesized for each of these regions comprising 93% of the wild type nucleotides and 2.33% of each of the other three nucleotides at amino acid codons wanted to

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low calcium positives from mutagenesis of aa 91-97 were sequenced.

The three other mutations (in codon 83, 103, 145), outside the 5 mutagenized region, can be explained by PCR misincorporation, although the mutation of S83T is a transversion which is quite unusual for PCR misincorporations.

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Table 2

	Strain number	Variant type					
5	59	I		G91A	N94K	D96A	
	60	II	S83T		N94K	D96N	
	61	II	S83T		N94K	D96N	
	62	III		E87K		D96V	
	63	IV		E87K	G91A	D96V	
10	64	II	S83T		N94K	D96N	
	65	III		E87K		D96V	
	67	V			N94K	F95L	D96H
	69	V			N94K	F95L	D96H
	71	III		E87K		D96V	
15	72	II	S83T		N94K	D96N	

Table 2: Strain number refers to the originally picked clones  
 20 cloned into *Aspergillus* expression vector pAHL. Variant type  
 refers to identical clones, which probably have arisen during  
 amplification of the random mutagenized library. Variant types  
 I and II are active in 0.01% Dobanol™25-7 while the rest are  
 inactive like wild type.

25

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## EXAMPLE 2

Analogously to the method described in Example 1, the following variants were constructed by random mutagenesis. The actual  
5 screening criteria used for selecting some of the variants are also described.

D167G+E210V

10 5mM EGTA, 0.01% Dobanol™25-7, 0.006% LAS  
E87K+G91A+L93I+N94K+D96A

5mM EGTA, 0.02% Dobanol™25-7  
N73D+S85T+E87K+G91A+N94K+D96A  
15 S83T+E87K+W89G+G91A+N94K+D96V  
E87K+G91A+D96R+I100V  
S83T+E87K+Q249R  
E87K+G91A

## 20 EXAMPLE 3

Expression of *Humicola lanuginosa* lipase in *Aspergillus oryzae*  
Cloning of *Humicola lanuginosa* lipase is described in EP 305  
216. It also describes expression and characterization of the  
25 lipase in *Aspergillus oryzae*. The expression plasmid used is  
named p960.

The expression plasmid used in this application is identical to  
p960, except for minor modifications just 3' to the lipase  
30 coding region. The modifications were made the following way:  
p960 was digested with NruI and BamHI restriction enzymes.  
Between these two sites the BamHI/NheI fragment from plasmid  
pBR322, in which the NheI fragment was filled in with Klenow  
polymerase, was cloned, thereby creating plasmid pA01 (figure  
35 2), which contains unique BamHI and NheI sites. Between these  
unique sites BamHI/XbaI fragments from p960 was cloned to give  
pAHL (figure 3).

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PCR Handle (=D): 5'-GGTCATCCAGTCACTGAGAC-3'

Helper 1 and helper 2 are complementary to sequences outside the coding region, and can thus be used in combination with any mutagenisation primer in the construction of a variant sequence.

All 3 steps are carried out in the following buffer containing:  
10 0.2 mM dATP, 0.2 mM dCTP, 0.2 mM dGTP, 0.2 mM TTP, 2.5 units Taq polymerase.

In step 1, 100 pmol primer A, 100 pmol primer B and 1 fmol linearized plasmid is added to a total of 100  $\mu$ l reaction mixture and 15 cycles consisting of 2 minutes at 95°C, 2 minutes at 37°C and 3 minutes at 72°C are carried out.

The concentration of the PCR product is estimated on an agarose gel. Then, step 2 is carried out. 0.6 pmol step 1 product and 1 fmol linearized plasmid is contained in a total of 100  $\mu$ l of the previously mentioned buffer and 1 cycle consisting of 5 minutes at 95°C, 2 minutes at 37°C and 10 minutes at 72°C is carried out.

25 To the step 2 reaction mixture, 100 pmol primer C and 100 pmol primer D is added (1  $\mu$ l of each) and 20 cycles consisting of 2 minutes at 95°C, 2 minutes at 37°C and 3 minutes at 72°C are carried out. This manipulation comprised step 3 in the mutagenisation procedure.

30

#### Isolation of mutated restriction fragment

The product from step 3 is isolated from an agarose gel and re-dissolved in 20  $\mu$ l H<sub>2</sub>O. Then, it is digested with the restriction enzymes BamHI and BstXI in a total volume of 50  $\mu$ l with the following composition: 100 mM NaCl, 50 mM Tris-HCl, pH 7.9, 10 mM MgCl<sub>2</sub>, 1 mM DTT, 10 units of BamHI and 10 units of BstXI. Incubation is at 37°C for 2 hours. The 733 bp BamHI/BstXI fragment is isolated from an agarose gel.

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pAHLA121V 5' -CCTTAACGTATCAACTACAGACCTCCA-3'  
 pAHLR205K/E210Q 5' -GCTGTAACCGAATTGGCGCGGGGAGCTTAGGG-  
 ACAATATC-3'  
 pAHLN73D/S85T/E87K/G91A/N94K/D96A  
 5 5' -TATTTCTTTCAAAGCGAACTTAAGATTAGCGATC-  
 CAGTTCTTTATAGTACGAGAGCCACGGAA-  
 AGAGAGGACGATCAATTTGTCCGTGTTGTCGAG-3'  
 pAHL83T/E87K/W89G/G91A/N94K/D96V  
 10 5' -TATTTCTTTCAAACCGAACTTAAGATTAGCGATA-  
 CCGTTCTTTATGGAACGAGTGCCACGGAAAGA-3'  
 pAHLE87K/G91A/D96R/I100V  
 5' -GCAAATGTCATTAACCTTCTTTCAATCTGAAGTTAA-  
 GATTAGCGATCCAGTTCTTTATGGAACGAGA-3'  
 pAHL83T/E87K 5' -CCCGATCCAGTTCTTTATGGAACGAGTGCCACGG-  
 15 AAAGA-3'  
 pAHLE87K/G91A 5' -GAAGTTAAGATTAGCGATCCAGTTCTTTATGGAA-  
 CGAGA-3'  
 pAHL83T/E87K 5' -CCCGATCCAGTTCTTTATGGAACGAGTGCCACGG-  
 AAAGA-3'  
 20 pAHLQ249R 5' -CGGAATGTTAGGTCTGTTATTGCCGCC-3'

## EXAMPLE 5

25 Construction of plasmids expressing combination analogues of *Humicola lipase*

The plasmids pAHLD167G/E210V  
 pAHLA121V/R205K/E210Q  
 and pAHL83T/E87K/Q249R

30

are constructed by performing two successive mutagenisation steps using the appropriate primers.

## 35 EXAMPLE 6

Expression of lipase analogues in *Aspergillus*

Transformation of *Aspergillus oryzae* (general procedure)

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7 days at 37°C spores are picked, suspended in sterile water and spread for single colonies. This procedure is repeated and spores of a single colony after the second reisolation are stored as a defined transformant.

5

#### Expression of lipase analogues in *A. oryzae*

The plasmids described above are transformed into *A. oryzae* IFO 4177 by cotransformation with p3SR2 containing the amdS gene from *A. nidulans* as described in the above example. Protoplasts  
10 prepared as described are incubated with a mixture of equal amounts of expression plasmid and p3SR2, approximately 5 µg of each are used. Transformants which could use acetamide as sole nitrogen source are reisolated twice. After growth on YPD for three days, culture supernatants are analyzed using an assay  
15 for lipase activity. The best transformant is selected for further studies and grown in a 1 l shake-flask on 200 ml FG4 medium (3% soy meal, 3% maltodextrin, 1% peptone, pH adjusted to 7.0 with 4 M NaOH) for 4 days at 30°C.

20 EXAMPLE 7

#### Purification of lipase variants of the invention

##### Assay for lipase activity :

25 A substrate for lipase was prepared by emulsifying glycerine tributyrat (MERCK) using gum-arabic as emulsifier.

Lipase activity was assayed at pH 7 using pH stat method. One  
unit of lipase activity (LU/mg) was defined as the amount  
30 needed to liberate one micromole fatty acid per minute.

Step 1:- Centrifuge the fermentation supernatant, discard the precipitate. Adjust the pH of the supernatant to 7 and add gradually an equal volume of cold 96 % ethanol. Allow the  
35 mixture to stand for 30 minutes in an ice bath. Centrifuge and discard the precipitate.

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- Method: 3 cycles with overnight drying between each cycle
- Wash liquor: 100 ml per beaker
- Swatches: 6 swatches (3.5 x 3.5 cm) per beaker
- 5 Fabric: 100% cotton, Test Fabrics style #400
- Stain: Lard coloured with Sudan red (0.75 mg dye/g of lard). 6  $\mu$ l of lard heated to 70°C was applied to the centre of each swatch. After application of the stain, the swatches were heated in an oven at 75°C for 30 minutes. The swatches were then stored overnight at room temperature prior to the first wash.
- 10
- Detergent:
- |                              |          |
|------------------------------|----------|
| LAS (Nansa 1169/P, 30% a.m.) | 1.17 g/l |
| AEO (Dobanol™25-7)           | 0.15 g/l |
| 15 Sodium triphosphate       | 1.25 g/l |
| Sodium sulphate              | 1.00 g/l |
| Sodium carbonate             | 0.45 g/l |
| Sodium silicate              | 0.15 g/l |
- pH: 10.2
- 20 Lipase conc.: 0.075, 0.188, 0.375, 0.75 and 2.5 mg of lipase protein per litre
- Time: 20 minutes
- Temperature: 30°C
- Rinse: 15 minutes in running tap water
- 25 Drying: overnight at room temperature (-20°C, 30-50% RH)
- Evaluation: after the 3rd wash, the reflectance at 460 nm was measured.

### 30 Results

Dose-response curves were compared for the lipase variants and the native *H. lanuginosa* lipase. The dose-response curves were calculated by fitting the measured data to the following equation:

$$35 \quad \Delta R = \Delta R_{\max} \frac{C^{0.5}}{K + C^{0.5}} \quad (I)$$

where  $\Delta R$  is the effect expressed in reflectance units

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Table 1

	Variant	Improvement factor
	E87K+D96V	1.2
	S83T+N94K+D96N	2.3
5	N94K+D96A	2.7
	E87K+G91A+D96A	2.6
	N94K+F95L+D96H	3.3
	D167G+E210V	5.0
10	E87K+G91A+L93I+N94K+D96A	1.3
	E87K+G91A+D96R+I100V	5.2
	E87K+G91A	5.0
	N73D+E87K+G91A+N94I+D96G	1.3
15	S83T+E87K+G91A+N92H+N94K+D96M	3.8
	K46R+E56R+G61S	1.9
	D102K	0.2
	D167G	1
20	N73D+E87K+G91A+N94I+D96G	1.3
	E210R	2.7
	E210K	5.5
	E210W	1
25	N251W+D254W+T267W	0.8
	S83T+E87K+G91A+N92H+N94K+D96M	3.8
	E56R+I90F+D96L+E99K	4.8
30	D57G+N94K+D96L+L97M	1.9

} random

} random

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Fowler et al., Molec. gen. Genet., 133, pp. 179-191, 1974.

Brady et al., "A Serine Protease Triad Forms the Catalytic Centre of a Triacylglycerol Lipase", Nature 343, 1990, pp. 767-  
5 770, 1990.

Tilbeyrgh, H. van, Egloff, M.-P., Martinez, C., Rugani, N.,  
Verger, R. and Cambillau (1993) Nature 362, p. 814-820.  
Interfacial activation of the lipase-prolipase complex by mixed  
10 micelles revealed by X-ray crystallography.

Hudson et al., Practical Immunology, Third edition, Blackwell  
Scientific Publications, 1989

15 Alber, T. and Kawasaki, G., J.Mol.Appl. Genet 1, 419-434 (1982)

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ATG AGG AGC TCC CTT GTG CTG TTC TTT GTC TCT GCG TGG ACG GCC TTG 48  
 Met Arg Ser Ser Leu Val Leu Phe Phe Val Ser Ala Trp Thr Ala Leu  
 -20 -15 -10  
 5 GCC AGT CCT ATT CGT CGA GAG GTC TCG CAG GAT CTG TTT AAC CAG TTC 96  
 Ala Ser Pro Ile Arg Arg Glu Val Ser Gln Asp Leu Phe Asn Gln Phe  
 -5 1 5 10  
 10 AAT CTC TTT GCA CAG TAT TCT GCA GCC GCA TAC TGC GGA AAA AAC AAT 144  
 Asn Leu Phe Ala Gln Tyr Ser Ala Ala Ala Tyr Cys Gly Lys Asn Asn  
 15 20 25  
 15 GAT GCC CCA GCT GGT ACA AAC ATT ACG TGC ACG GGA AAT GCC TGC CCC 192  
 Asp Ala Pro Ala Gly Thr Asn Ile Thr Cys Thr Gly Asn Ala Cys Pro  
 30 35 40  
 20 GAG GTA GAG AAG GCG GAT GCA ACG TTT CTC TAC TCG TTT GAA GAC TCT 240  
 Glu Val Glu Lys Ala Asp Ala Thr Phe Leu Tyr Ser Phe Glu Asp Ser  
 45 50 55  
 20 GGA GTG GGC GAT GTC ACC GGC TTC CTT GCT CTC GAC AAC ACG AAC AAA 288  
 Gly Val Gly Asp Val Thr Gly Phe Leu Ala Leu Asp Asn Thr Asn Lys  
 60 65 70  
 25 TTG ATC GTC CTC TCT TTC CGT GGC TCT CGT TCC ATA GAG AAC TGG ATC 336  
 Leu Ile Val Leu Ser Phe Arg Gly Ser Arg Ser Ile Glu Asn Trp Ile  
 75 80 85  
 30 GGG AAT CTT AAC TTC GAC TTG AAA GAA ATA AAT GAC ATT TGC TCC GGC 384  
 Gly Asn Leu Asn Phe Asp Leu Lys Glu Ile Asn Asp Ile Cys Ser Gly  
 95 100 105  
 35 TGC AGG GGA CAT GAC GGC TTC ACT TCG TCC TGG AGG TCT GTA GCC GAT 432  
 Cys Arg Gly His Asp Gly Phe Thr Ser Ser Trp Arg Ser Val Ala Asp  
 110 115 120  
 35 ACG TTA AGG CAG AAG GTG GAG GAT GCT GTG AGG GAG CAT CCC GAC TAT 480  
 Thr Leu Arg Gln Lys Val Glu Asp Ala Val Arg Glu His Pro Asp Tyr  
 125 130 135  
 40 CGC GTG GTG TTT ACC GGA CAT AGC TTG GGT GGT GCA TTG GCA ACT GTT 528  
 Arg Val Val Phe Thr Gly His Ser Leu Gly Gly Ala Leu Ala Thr Val  
 140 145 150  
 45 GCC GGA GCA GAC CTG CGT GGA AAT GGG TAT GAT ATC GAC GTG TTT TCA 576  
 Ala Gly Ala Asp Leu Arg Gly Asn Gly Tyr Asp Ile Asp Val Phe Ser  
 155 160 165 170  
 50 TAT GGC GCC CCC CGA GTC GGA AAC AGG GCT TTT GCA GAA TTC CTG ACC 624  
 Tyr Gly Ala Pro Arg Val Gly Asn Arg Ala Phe Ala Glu Phe Leu Thr  
 175 180 185  
 55 GTA CAG ACC GGC GGA ACA CTC TAC CGC ATT ACC CAC ACC AAT GAT ATT 672  
 Val Gln Thr Gly Gly Thr Leu Tyr Arg Ile Thr His Thr Asn Asp Ile  
 190 195 200  
 55 GTC CCT AGA CTC CCG CCG CGC GAA TTC GGT TAC AGC CAT TCT AGC CCA 720  
 Val Pro Arg Leu Pro Pro Arg Glu Phe Gly Tyr Ser His Ser Ser Pro  
 205 210 215  
 60 GAG TAC TGG ATC AAA TCT GGA ACC CTT GTC CCC GTC ACC CGA AAC GAT 768  
 Glu Tyr Trp Ile Lys Ser Gly Thr Leu Val Pro Val Thr Arg Asn Asp  
 220 225 230  
 65 ATC GTG AAG ATA GAA GGC ATC GAT GCC ACC GGC GGC AAT AAC CAG CCT 816  
 Ile Val Lys Ile Glu Gly Ile Asp Ala Thr Gly Gly Asn Asn Gln Pro  
 235 240 245 250  
 AAC ATT CCG GAT ATC CCT GCG CAC CTA TGG TAC TTC GGG TTA ATT GGG 864

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Ile Val Lys Ile Glu Gly Ile Asp Ala Thr Gly Gly Asn Asn Gln Pro  
235 240 245 250  
5 Asn Ile Pro Asp Ile Pro Ala His Leu Trp Tyr Phe Gly Leu Ile Gly  
255 260 265  
Thr Cys Leu

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6. The method according to claim 5, in which the host cell is a cell of a fungal or a bacterial strain.
7. The method according to claim 6, in which the host cell is a  
5 cell of the genus *Aspergillus*, such as *A. niger*, *A. oryzae* and *A. nidulans*, or a cell of the genus *Saccharomyces*, e.g. *S. cerevisiae*.
8. The method according to claim 6, in which the host cell is a  
10 cell of a gram-positive bacterial strain, e.g. of the genus *Bacillus*, such as *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus lentus*, *Bacillus brevis*, *Bacillus stearothermophilus*, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus coagulans*, *Bacillus circulans*, *Bacillus lautus*, *Bacillus*  
15 *thuringiensis* or *Streptomyces lividans* or *Streptomyces murinus*, or a cell of a gram-negative bacterial strain, such as *E. coli*.
9. The method according to claim 1, in which the mutated lipolytic enzyme has an improved tolerance towards a non-ionic,  
20 anionic, kationic, zwitterionic or amphoteric surfactant.
10. The method according to claim 9, in which the non-ionic surfactant is an alcohol ethoxylate and/or the anionic surfactant is LAS or an alkyl sulphate.  
25
11. The method according to claim 1, wherein host cells screened in step (c) are subjected to a second mutagenesis treatment, to rescreening, to reisolation and/or to recloning.
- 30 12. The method according to any of claims 1-11, in which the random mutagenesis is localized to a part of the DNA sequence encoding the parent lipolytic enzyme.
13. The method according to any of claims 1-12, in which the  
35 parent lipolytic enzyme is a lipase, an esterase, a cutinase or a phospholipase.

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22. The method according to claim 16, wherein the parent lipolytic enzyme is derivable from a bacterium.

23. The method according to claim 22, wherein the DNA sequence  
5 encoding the parent lipolytic enzyme is derivable from a strain of *Pseudomonas* spp., such as *P. cepacia*, *P. alcaligenes*, *P. pseudoalcaligenes* or *P. fragi*. or from a strain of *Bacillus*.

24. A variant of a lipolytic enzyme prepared by the method  
10 according to any of claims 1-23.

25. A variant according to claim 24 which is a variant of the *H. lanuginosa* lipase obtainable from DSM 4109 or a analogue thereof, which comprises a mutation in at least one of the following  
15 positions:

K46, E56, S58, G61, T64, N73, S83, I90, G91, N92, N94, D96, L97,  
K98, E99, I100, D102, A121, E129, D167, R205, E210, K237, N251,  
I252, D254, P256, G263, L264 or T267.

20

26. A variant of the *H. lanuginosa* lipase obtainable from strain DSM 4109 or a analogue of said lipase, which carries a mutation in at least one of the regions defined by the amino acid residues 56-64, 83-100 or 205-211.

25

27. A variant according to claim 26, which comprises at least one of the following mutations:

K46R, D57G, S58F, G61S, D62C, T64R, S83T, I90F, G91A, N92H, N94I,  
30 N94K, L97M, K98I, I100V, D102K, A121V, E129K, D167G, R205K,  
E210W, K237M, N259W, I252L, D254W, P256T, G263A, L264Q or T267W.

28. A variant of the *H. lanuginosa* lipase obtainable from DSM 4109 or an analogue thereof comprising at least one of the  
35 following mutations:

N94K+D96A

S83T+N94K+D96N

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30. A DNA construct encoding a *H. lanuginosa* lipase variant according to any of claims 24-28.
31. A vector harbouring a DNA construct according to claim 29 or  
5 30.
32. The vector according to claim 31, which is a plasmid or a bacteriophage.
- 10 33. The vector according to claim 31 or 32, which is an expression vector further comprising DNA sequences permitting expression of the variant of the parent lipolytic enzyme.
34. A host cell harbouring a DNA construct according to claim 29  
15 or 30 or a vector according to any of claims 31-33.
35. The cell according to claim 34, which is a microbial cell.
36. The cell according to claim 35, which is a cell of a fungal  
20 or a bacterial strain.
37. The cell according to claim 36, which is a cell of the genus *Aspergillus*, such as *A. niger*, *A. oryzae* and *A. nidulans*, or a cell of the genus *Saccharomyces*, e.g. *S. cerevisiae*.  
25
38. The cell according to claim 36, which is a cell of a gram-positive bacterial strain, e.g. of the genus *Bacillus*, such as *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus lentus*, *Bacillus brevis*, *Bacillus stearothermophilus*, *Bacillus*  
30 *alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus coagulans*, *Bacillus circulans*, *Bacillus lautus*, *Bacillus thuringiensis* or *Streptomyces lividans* or *Streptomyces murinus*, or a cell of a gram-negative bacterial strain, such as *E. coli*.
- 35 39. A method of producing a variant of a parent lipolytic enzyme which has a decreased dependance to calcium and/or an improved tolerance towards a detergent or a detergent component as compared to the parent lipolytic enzyme, which method comprises

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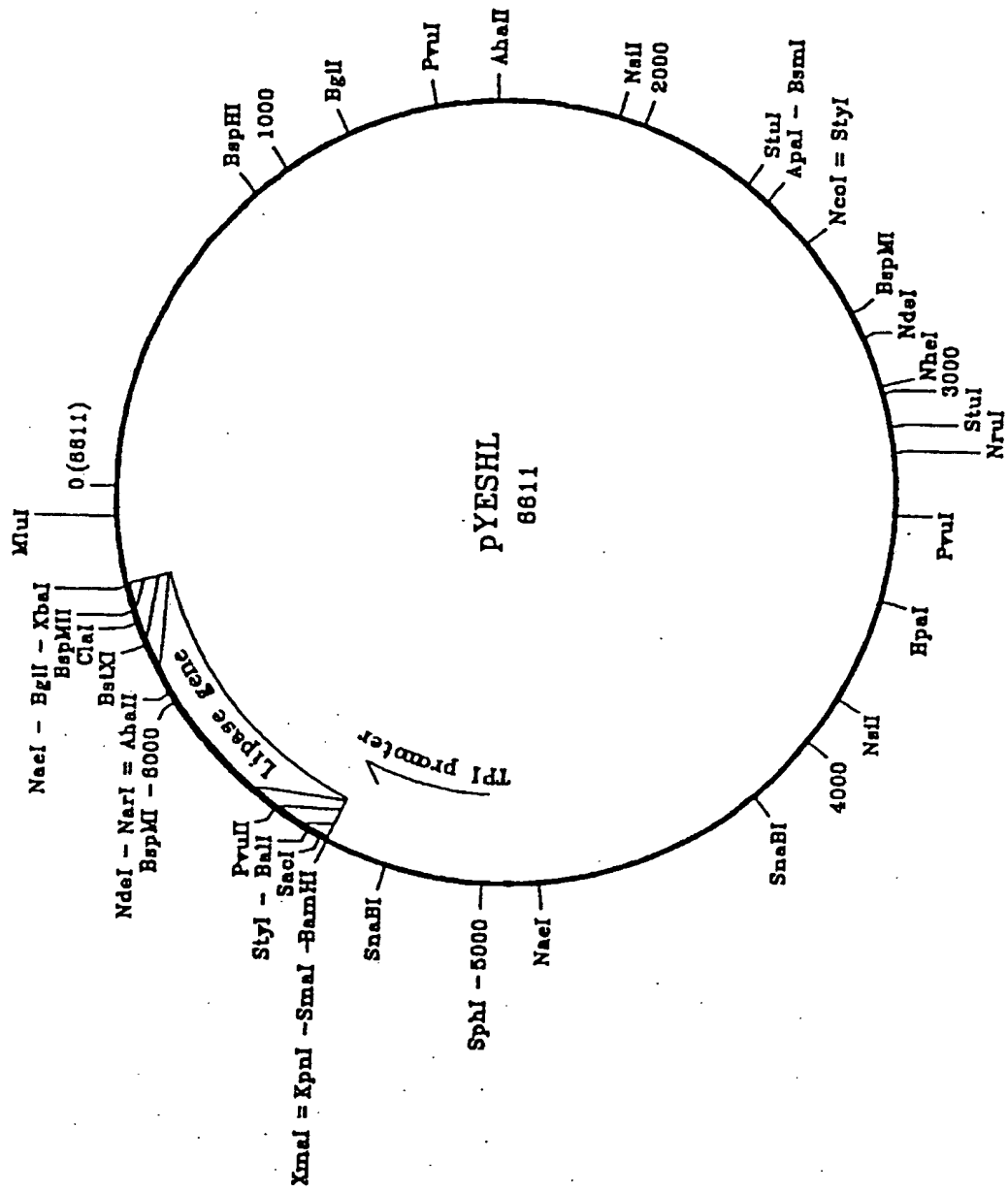


Fig. 1



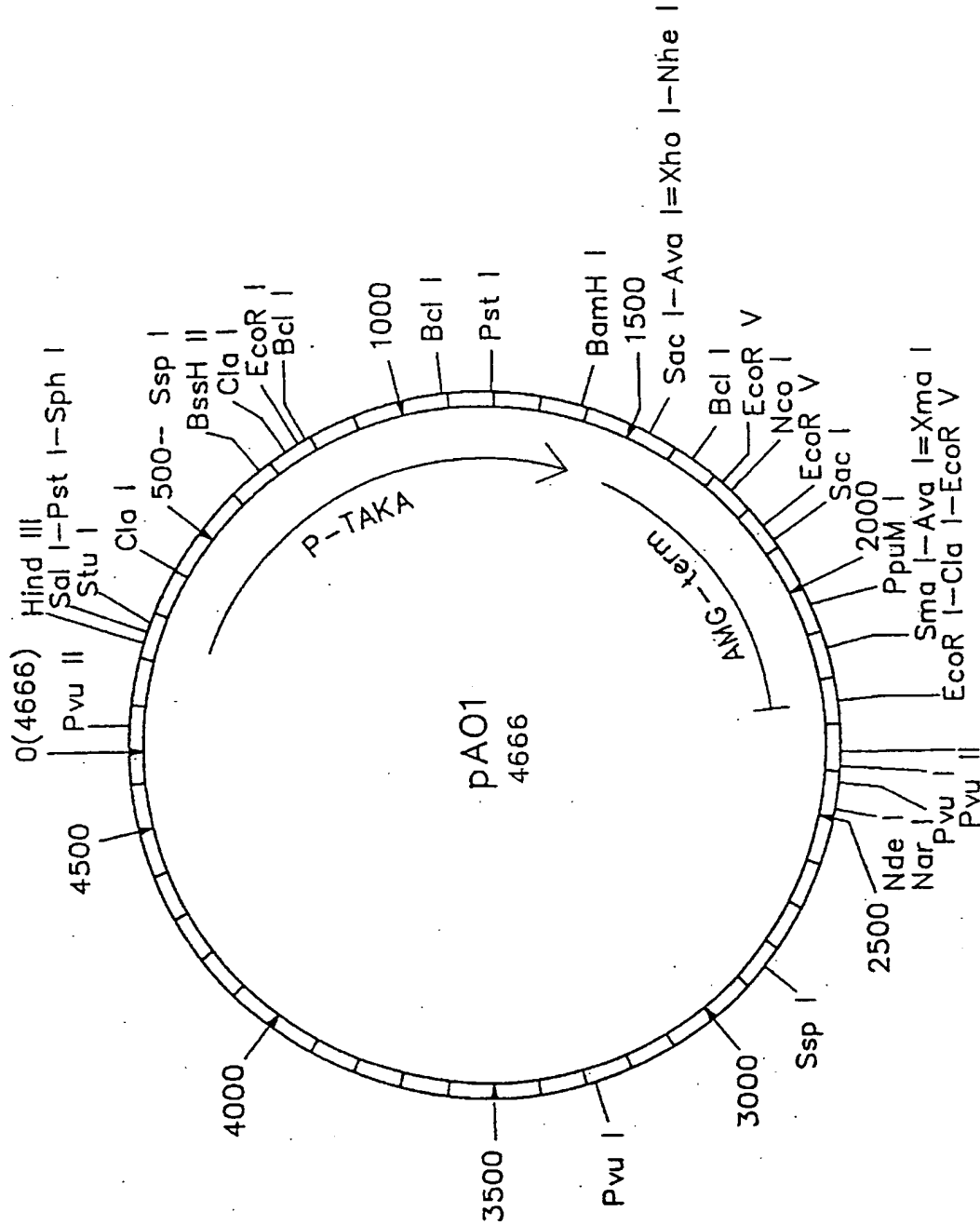


Fig. 2

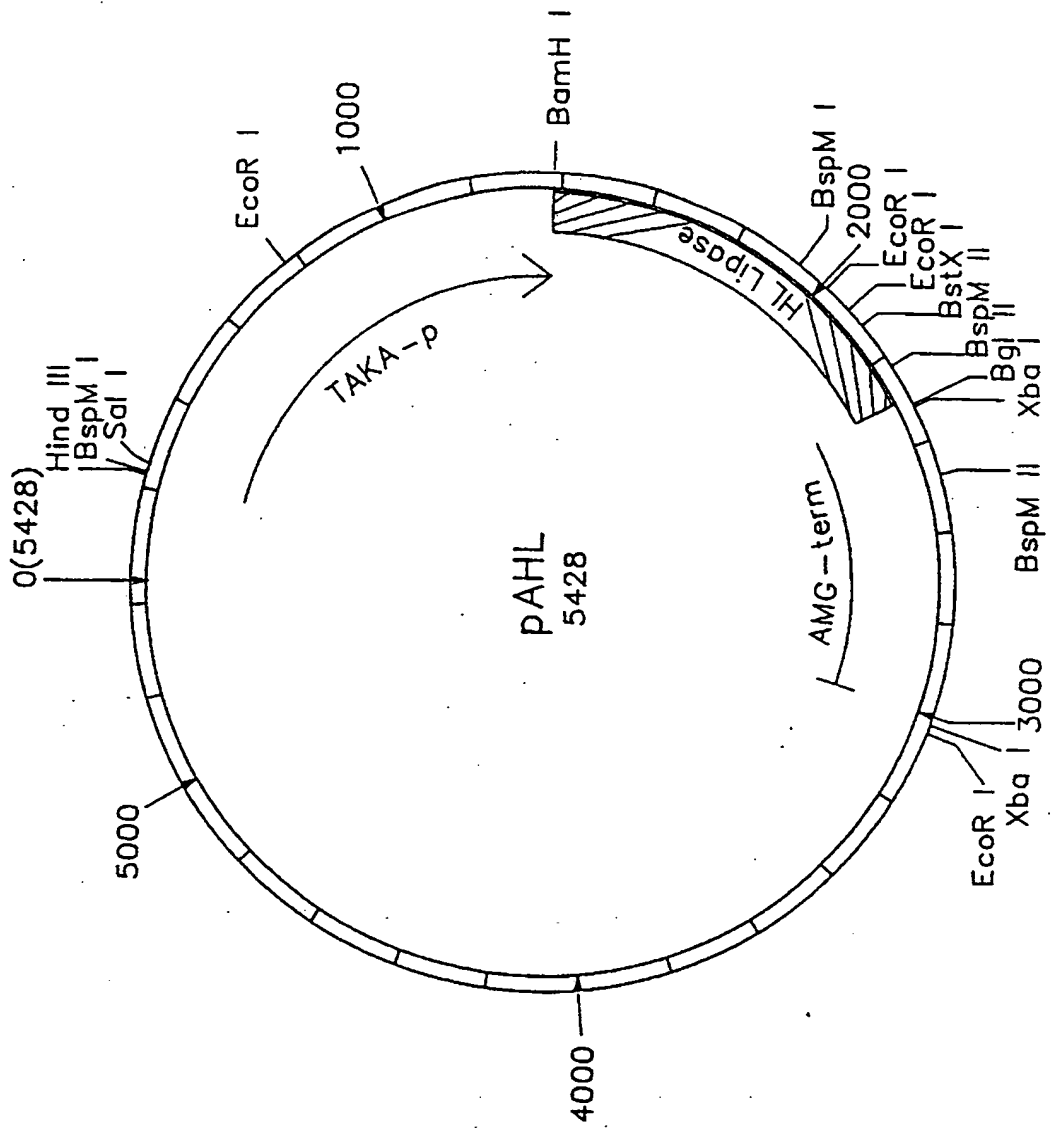


Fig. 3

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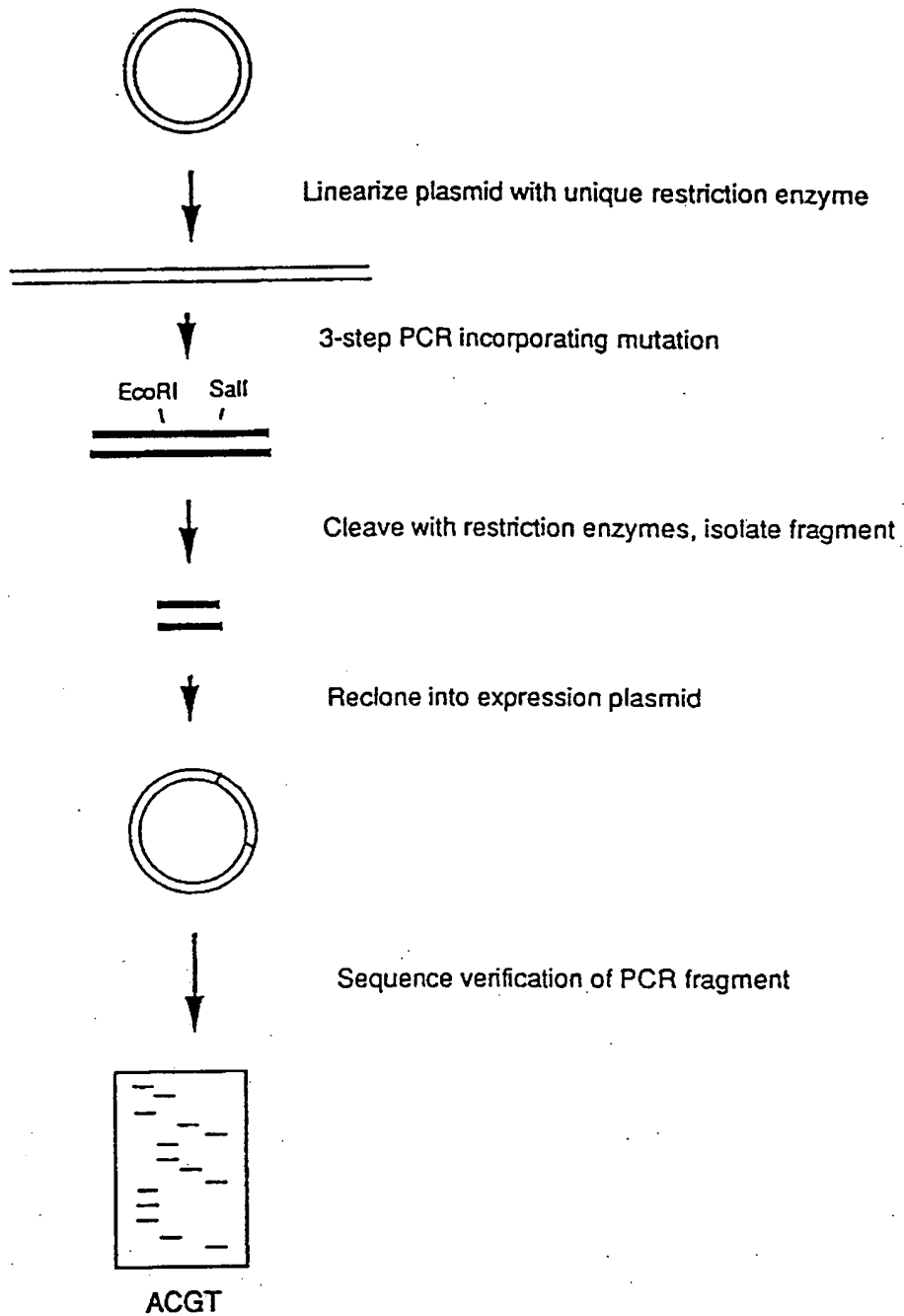


Fig. 4

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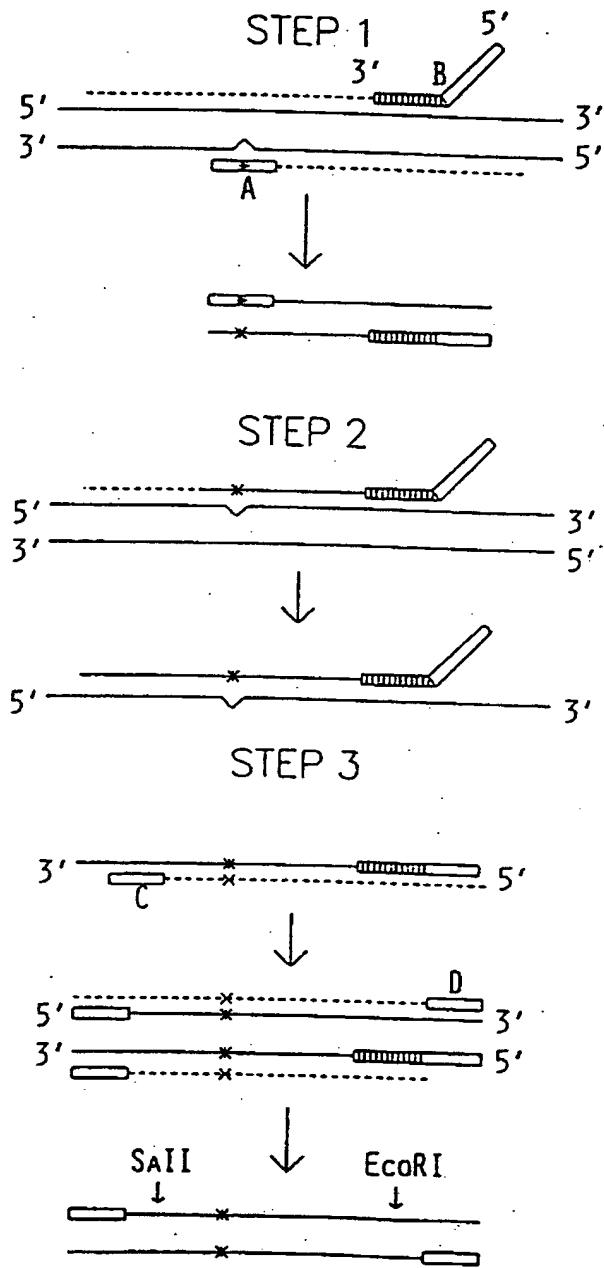


Fig. 5

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 95/00079

<b>A. CLASSIFICATION OF SUBJECT MATTER</b>		
IPC6: C12N 15/55, C12N 9/20, C11D 3/386 According to International Patent Classification (IPC) or to both national classification and IPC		
<b>B. FIELDS SEARCHED</b>		
Minimum documentation searched (classification system followed by classification symbols)		
IPC6: C12N		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
SE,DK,FI,NO classes as above		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)		
MEDLINE, BIOSIS, WPIL, CA		
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP, A2, 0525610 (SOLVAY ENZYMES GMBH & CO. KG), 3 February 1993 (03.02.93), the claims --	1-46
X	WO, A1, 9205249 (NOVO NORDISK A/S), 2 April 1992 (02.04.92), page 2, line 22 - page 3, line 14; page 18, line 23 - line 32, the claims --	1-46
P,X	WO, A1, 9414964 (UNILEVER N.V.), 7 July 1994 (07.07.94), page 9, line 4 - line 11, the claims --	1-46
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input checked="" type="checkbox"/> See patent family annex.		
<ul style="list-style-type: none"> <li>* Special categories of cited documents:</li> <li>*A* document defining the general state of the art which is not considered to be of particular relevance</li> <li>*E* earlier document but published on or after the international filing date</li> <li>*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</li> <li>*O* document referring to an oral disclosure, use, exhibition or other means</li> <li>*P* document published prior to the international filing date but later than the priority date claimed</li> <li>*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</li> <li>*X* document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</li> <li>*Y* document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</li> <li>*&amp;* document member of the same patent family</li> </ul>		
Date of the actual completion of the international search		Date of mailing of the international search report
13 June 1995		19 -06- 1995
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86		Authorized officer  Carolina Palmcrantz Telephone No. +46 8 782 25 00

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NZAS-0018145

**INTERNATIONAL SEARCH REPORT**

Information on patent family members

03/05/95

International application No.

PCT/DK 95/00079

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A2- 0525610	03/02/93	DE-A- 4224125	28/01/93
WO-A1- 9205249	02/04/92	AU-B- 657278	09/03/95
		AU-A- 8617291	15/04/92
		CA-A- 2092615	14/03/92
		EP-A- 0548228	30/06/93
		JP-T- 6501153	10/02/94
WO-A1- 9414964	07/07/94	NONE	

Form PCT/ISA/210 (patent family annex) (July 1992)

NZAS-0018146